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4. Analysis of marine poisons and the applications

(Seminar / The latest techniques in the analysis of foodstuffs)

by Takeshi YASUMOTO (Tokyo University, Agriculture Department)

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It has recently become known that numerous fish and shellfish accumulate poisons produced by bacteria and microalgae and that they have so in some cases been the cause of food poisoning. There is concern that some of these compounds may if ingested for a long period of time be toxic to the internal organs and accelerate the occurrence of cancer, even though they are ingested below levels bringing about an acute condition. In the past, one used almost always in the examinations and quantitative analyses of these fish and shellfish poisons determinations based on mouse lethal toxicity. However, as international trade widened, it became imperative to standardize in the various countries the poison determination methods. In numerous countries of Europe and America the use of experimental animals for the purpose of determination has been seriously restricted or even forbidden, and researchers have been urged

to switch to instrumental analysis methods, immunity determination methods or to methods employing cultured cell systems as common determination method. Regarding the immunity determination methods, there exist numerous homologues with resembling structures, and the test results are difficult to evaluate when the specific activities differ. With cultured cell systems, the problems are the maintenance and the quantitative determination characteristics of the cultured cells. It is therefore urgent to develop quantitative determination methods combining fluorescent reaction and high-speed liquid chromatography (HPLC). In the present article, we wish to present simply the fish and shellfish poisons and provide explanations focussing on typical fluorescence - HPLC analysis methods usable for the analysis of the above poisons. For the purpose of application of these methods we also wish to focus on actual examples like the study of the food chain and the discovery of homologues.

Blowfish poison

The number of people dying from blowfish poisoning in Japan has recently decreased to about 10-20 per year. In fact, blowfish-caused deaths represents about 80% of the deaths due to poisoning by natural poison. The edible fish species and their edible parts are determined. However, toxicity shows individual and regional differences. Moreover, imported blowfish is on the increase so that a toxicity watch is indispensable. Meanwhile, the export of part of the blowfish produced by Japan to Washington D.C. in the US has now been authorized, so here too inspection has become necessary.

Tetrodotoxin (Fig. 1) which is the main substance causative of blowfish poisoning is a violent poison. The smallest lethal amount based on mouse peritoneal injection is 10 μg/kg. The time before death is as short as 30 min. The present official method for calculating the poison volume is to inject mice peritoneally with an extract, and to measure the time until death. So far there was no suitable fluorescent reagent for tetrodotoxin. Noting that Mosher et al who became famous for their discovery of newt tetrodotoxin performed heating after atomizing a caustic soda solution for detection by thin-layer chromatography and made detections in the mode of fluorescent spots, we built a postcolumn reaction system analyzer combined with HPLC. At first, we used ion exchange resin for the separation column but changed later to the ion pair method based on reverse-phase column and on a combination with heptafluoro acetic acid(1). The smallest detection volume based on this analyzer was 10 ng (3 pM).

The analyzer demonstrated for the first time that besides tetrodotoxin, blowfish contained also 4-epitetrodotoxin, 4,9 - anhydrotetrodotoxin. Another important point is that by taking advantage of the high sensitivity and specificity of the present analytical method, we were for the first time able to follow up the food chain of the organisms holding tetrodotoxin and to prove that the source of tetrodotoxin is a bacterium. When we later analyzed newts holding tetrodotoxin, we tried to understand why the poison volume calculated from the fluorescence intensity did not agree with the mouse toxicity, and we discovered 6-epitetrodotoxin, 11-deoxytetrodotoxin(2). We also

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discovered in kusa blowfish,* the above compounds with the addition of 11-nortetrodotoxin-6 (R) - ol[3]. One further discovered 11-OXO tetrodotoxin in blowfish from the southern seas. There are relatively large amounts of these homologues in blowfish from the southern seas. More particularly, the fluorescent strength per mol of 6-epitetrodotoxin is 8 times that of tetrodotoxin, and as it yielded the same retention time as tetrodotoxin with conventional analyzers it constituted an important factor of error in the analysis of blowfish from the southern seas. Keeping these points in mind, we recently improved the analysis conditions[4]. We show the outline as well as some typical chromatograms in the Figs. 2 and 3.

On the basis of the structure of the homologue group discovered through the use of the analyzer (Fig. 1) tetrodotoxin was biosynthesized from an arginine and isoprene unit and imagined to be changed into the various derivatives in an oxidative fashion (Fig. 4). In this manner, the use of an analyzer did not only replace the animal test method by an instrumental analysis method but it became also linked to the discovery of a powerful compound group suggesting a metabolic route. First, we believed that the substance of the fluorescent compounds produced when heating the tetrodotoxin together with alkali was 2-amino-6 hydroxymethyl - 8-hydroxyquinazolin known under the name of C9 base, but later noted that the present compound had a low fluorescent power and that the substance of the fluorescent matter was made from several unstable

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*Translator's note: Fugu niphobles (Jordan & Snyder)

components. We were not able to confirm the structure of these fluorescent matters as one would have had to degrade large volumes of tetrodotoxin. p. 403

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Paralytic shellfish poison

For a long time the countries of Europe and America, and mainly the people of the North American continent have known cases of food poisoning occurrences caused by bivalves in which the poison produced by the poisonous protogonyaulax plankton genus (former name: gonyaulax) had accumulated. The high mortality rate was similar to that caused by blowfish poisoning. The poison was feared under the name of paralytic shellfish poison. The number of poisoning deaths in North America is reported to have reached 2.000. In Japan there were 95 poisoning cases including 3 deaths. At the present time a watch has been called for in the entirety of Japan and poisoning occurrences are being prevented. Yet, poisoned shellfish has been noted in numerous areas. The shipping and distribution of shellfish exceeding the tolerance level has been prohibited and so the losses suffered by the shellfish farmers are very serious.

The main so far isolated toxins are shown in Fig. 5. Saxitoxin is the first causative toxin whose structure was determined. Similarly to the tetrodotoxin of the blowfish poison it is a water-soluble compound of small molecules numerous in hetero atoms. The smallest lethal amount based on mouse peritoneal administration is 9 µg/kg. The toxin resembles p. 404

also tetrodotoxin in that it has a guanidium base and an action of blocking the sodium channels of the cell walls. The present determination method consists in administering extract to mice peritoneally and to measure the survival time. The tolerable amount is set at 80 μg or less per 100 g of food.

The first test of chemical determination method was made by Rapoport et al who treated saxitoxin with hydrogen peroxide in an alkali solution and who performed detection by converting to the fluorescent compounds shown in Fig. 6[5]. However, the study led nowhere as the rate of change to the fluorescent mode was low among the numerous homologues discovered later in the group having an hydroxyl group at N of the position 1 such as neosaxitoxin, and as the separation of numerous homologues was difficult. However, great progress toward practical application was made since the time when Sullivan et al[6] proposed a pair ion separation method based on oxidation conditions employing periodic acid and a reverse-phase system column. Our colleague Oshima added a detailed examination of the oxidation and separation conditions and succeeded in the analytical determination of all components[7]. Fig. 7 and Fig. 8 show an outline of the apparatus and the chromatogrammes. As indicated in Fig. 5, the causative toxins are divided into 3 groups, that is one group holding two sulfuric acid radicals, another holding one radical and another one holding none. As the basic characteristics are greatly different, we use 3 types of solution system for the analyses. Moreover, as the strength of the fluorescent compounds increases under acid conditions, we add acid to the oxidation reaction. By employing this apparatus, we obtained a

sensitivity 10-10,000 times superior to that obtained by using mice. The minimum detection amount depends on the components but is nevertheless within the 1-100 ng range. One may say that exception being made for some particular samples, a quite practical stage has been reached. The present analyzer allows also to pursue the study of the metabolic course and to discover new components, and is highly regarded for tracking the food chain.

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Diarrhetic shellfish poison p. 405

We showed that the mussel-caused food poisoning diarrheas that in 1977 broke out in the Miyagi Prefecture were not caused by bacterial contamination but by natural poison. This was called diarrhetic shellfish poison. This type of poisoning broke out repeatedly later in the country, and up to 10,000 poisoning cases have been reported in the recent years along the Atlantic coasts of Europe. Important bivalves such as mussels, short-necked clams, scallops are poisonous every year for a long period of time, and there are shipping regulations. The mouse peritoneal administration method used as official method is inconvenient on all counts of precision, sensitivity, particularity and required analysis time, and one is looking forward to instrumental analysis methods. In order to know the chemical structure needed to design an analysis method, we performed extraction from the midgut gland of scallops and determined the structure by isolating the polyether group shown in Fig. 9(8).

The first isolated dinophysistoxins-1, -3 (DTS_1, DTX_3) were derivatives of the compound called okadaic acid (OA) previously isolated from kuroiso sponge*. Later OA itself was isolated from mussels in Europe. This group is strongly causative of diarrhea and is also a cancer promoter. The second group, that is, the macrolide compounds, the pectenotoxin group (PTXs) does not cause diarrhea but is toxic to the liver. The action of the third type viz. Yessotoxins (YTX) is not yet clear. The tolerable amount in foodstuff is specified in terms of mouse toxicity. If the value is converted to DTX_1 case, the amount should not be more than 180 ng in 1 g of food. If among the component shown in Fig. 9, those components having a carboxyl radical are allowed to react against 9-anthryl diazomethane (Funakoshi Pharmaceuticals) they become fluorescent and we established a method of determination by HPLC(9). The midgut glands of the bivalves used as samples contain numerous interfering matters represented mainly by fatty acids. So, one must perform pretreatment by a solvent fraction prior to the reaction and remove the interfering matters with a small-capacity cartridge column after the reaction. Fig. 10 shows the reaction system and the chromatogrammes. The smallest amount detected was 100 pg. With regard to the pectenotoxin group, we tried to label the common 14th position ketone with fluorescence but the reaction did not progress on account of steric hindrance. For PTX, it was possible to use 1-anthroyl ** nitrile(10) which had been developed as a fluorescence-producing

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Translator's notes:
* Halichondria okadai (Halichondriiae)
** "ansuroiru" as transliterated

reagent made of alcoholic hydroxyl groups[11]. Fig. 11 shows the reaction system and the chromatogrammes. No trace volume instrumental analysis method has been developed for yessotoxin yet.

Thus one has not yet reached practical means for determining all components through instrumental analysis as the composition of the diarrhetic shellfish poison of the Japanese bivalves is quite complex. However, as the toxin component in most European regions is okadaic acid only, the fluorescence-HPLC method using 9-anthryl diazomethane is effective and is in the process of being used practically. The problem is in the developing of an appropriate internal standard. Meanwhile, the fluorescence-HPLC analysis method was useful in pursuing the study of the food chain. It is believed that the reason why the shellfish becomes poisonous with diarrhetic shellfish poison is that the monocellular phytoplankton Dinophysis fortii produces the poison and that the bivalves ingest this plankton and accumulate the poison. However, this species of plankton is an heterotrophic species and cannot be cultivated artificially. As the appearance density in seawater is low, it is impossible to secure the amount needed to confirm the power of toxicity production, and confirming the toxicity has been a subject of concern for a long time. We treated seawater with a sieve and concentrated the Dinophysis. After this, we operated a capillary tube under microscope and collected one at a time cells in pure condition. As a result we were able to detect and confirm DTX_1 and PTX_2 through the analysis of the obtained samples[12]. We were also able to detect and identify DTX_1 and okadaic acid in 7 other species of Dinophysis and to confirm that they were species causative of poisoning.

The reason why we added in this series a paragraph about the analysis of marine poisons is not that we wanted to present analytical methods specially different from those used for other biocomponents. There is a strong tendency to rely on animal tests in the analysis not only of marine poisons but of all poisonous substances. By endeavouring to develop an instrumental analysis method of high sensitivity and specificity, we wanted to show by some examples that it was possible to follow the food chain and the metabolic products. We also presented two examples, one of a postcolumn reaction of compounds for which there are no suitable fluorescence-production reagents and another of a precolumn method using commercial fluorescence reagents. We are also presently trying various methods for the other poisonous components and we believe that in the near future it will be possible to perform instrumental analyses of most marine poisons.

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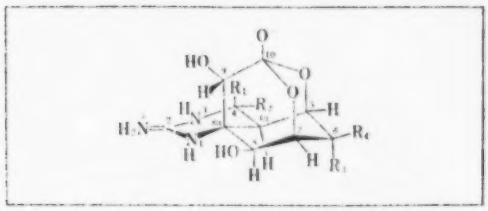

		R_1	R_2	R_3	R_4
a	テトロドトキシン	H	OH	OH	CH_2OH
b	4-エピテトロドトキシン	OH	H	OH	CH_2OH
c	6-エピテトロドトキシン	H	OH	CH_2OH	OH
d	11-デオキシテトロドトキシン	H	OH	OH	CH_3
e	11-オキソテトロドトキシン	H	OH	OH	CHO
f	11-ノルテトロドトキシン-6-(R)-オール	H	OH	H	OH

図 1■フグから発見されたテトロドトキシン(TTX)関連化合物の構造

Fig. 1 Structure of tetrodotoxin (TTX)-related compounds discovered in blow fish

a tetrodotoxin
b 4- epitetrodotoxin
c 6- epitetrodotoxin
d 11- deoxytetrodotoxin
e 11 oxotetrodotoxin
f 11 nortetrodotoxin - 6 - (R) - ol

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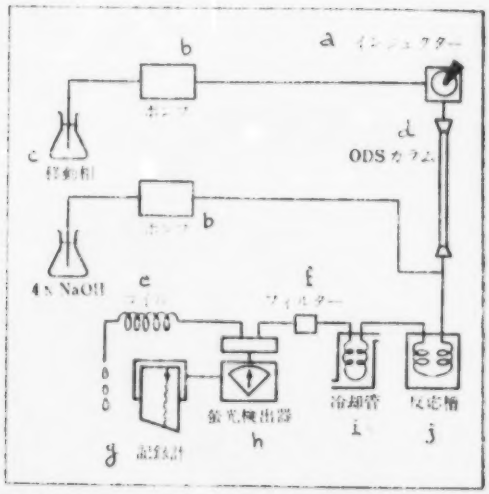

図 2■テトロドトキシンとその同族体の分析装置

Fig. 2 Analyzer for tetrodotoxin and its homologues

a injector
b pump
c mobile phase
d column
e coil
f filter
g recorder
h fluorescence detector
i cooling pipe
j reaction tank

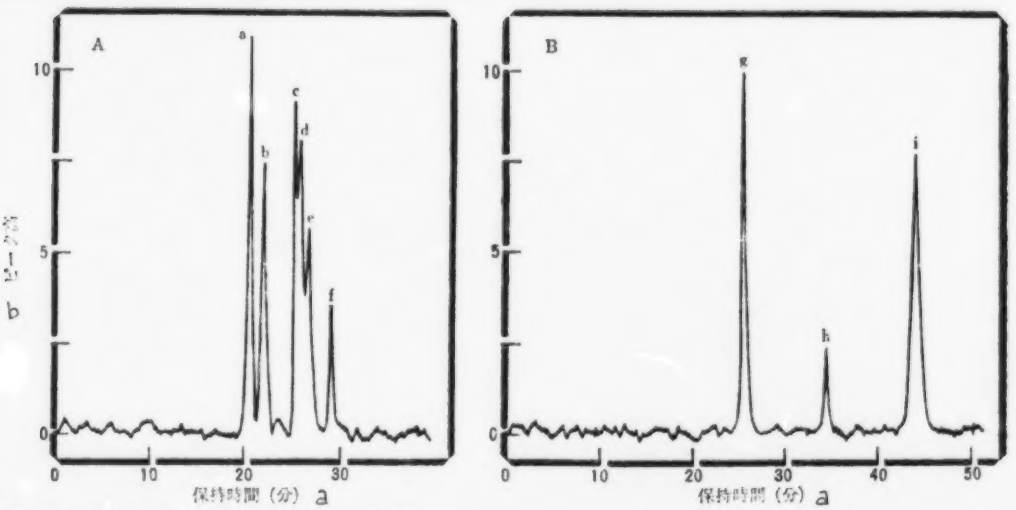

図 3■テトロドトキシン同族体のクロマトグラム

c カラム：Develosil ODS（野村化学，1×25cm），溶媒：0.006N ヘプタフルオロ酢酸含有 0.001N 酢酸アンモニウム緩衝液（pH 5.0），クロマトグラム A：反応温度，135℃，検出 ex. 375nm，ex. 500nm．クロマトグラム B：反応温度，180℃，検出 ex. 412nm，em. 517nm．

d 標品混合物 A：テトロドトキシン（a），6-エピテトロドトキシン（b），4,9-アンヒドロテトロドトキシン（c），11-ノルテトロドトキシン-6-(*R*)-オール（d），4-エピテトロドトキシン（e），4,9-アンヒドロテトロドトキシン（f），B：11-デオキシテトロドトキシン（g），4-エピ-11-デオキシテトロドトキシン（h），4,9-アンヒドロ-11-デオキシテトロドトキシン（i）

Fig. 3 Chromatogrammes of tetrodotoxins homologues

a retention time (min)
b peak height
c column: Develosil ODS (Nomura Kayaku, 1 X 25 cm), solvent: 0.006 N heptafluoroacetic acid-containing 0.001 N ammonium acetate buffer solution (pH 5.0), chromatogramme A: reaction temperature 135°C, detection: ex. 375 nm, ex. 500 nm. Chromatogramme B: reaction temperature 180°C, detection ex. 412 nm, em. 517 nm.

d Specimen mixtures A: tetrodotoxin (a), 6-epitetrodotoxin (b), 4.9 anhydrotetrodotoxin (c), 11-nortetrodotoxin-6-(R)-ol (d), 4-epitetrodotoxin (e), 4-9 anhydrotetrodotoxin (f), B: 11-deoxytetrodotoxin (g), 4-epi-11-deoxytetrodotoxin (h), 4-9 anydro-11-deoxytetrodotoxin (i).

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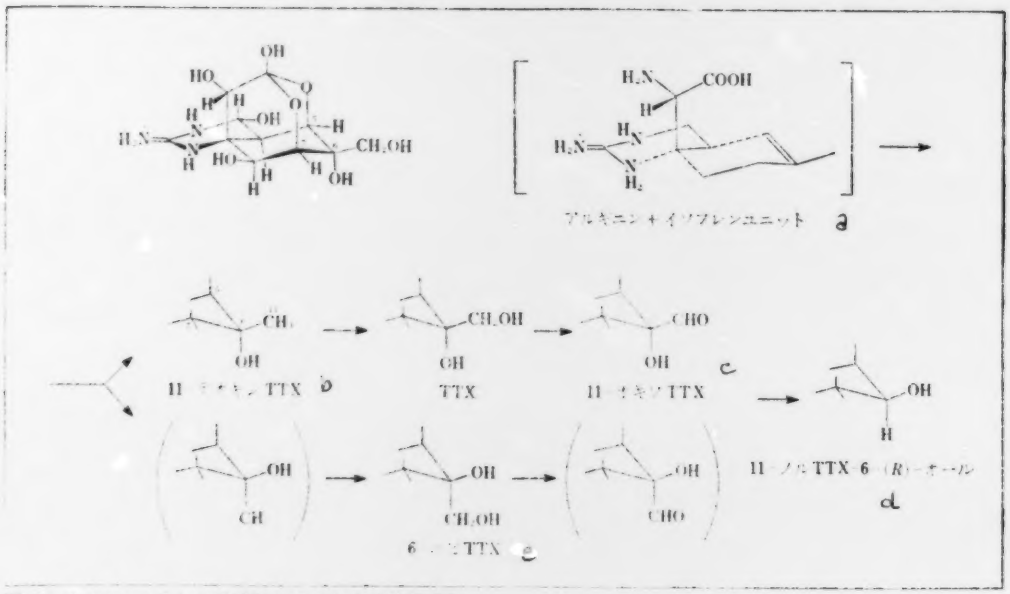
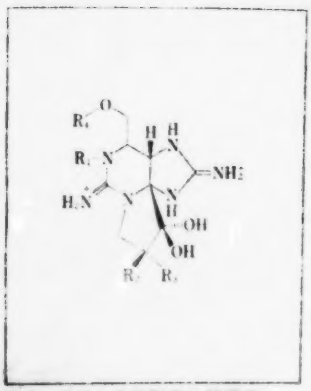


図 4■テトロドトキシンの推定生合成および代謝経路
( ) 内は未検出

Fig. 4 Estimated biosynthesis and metabolic route of the tetrodotoxins

( ): undetected

a alginine + isoprene unit
b 11- deoxy TTX
c 11- oxo-TTX
d 11 11- nol TTX-6-(R)-ol
e 6- epi TTX



R_1	R_2	R_3	R_4	
H	H	H	$CONH_2$	a サキシトキシン (STX)
OH	H	H	$CONH_2$	b ネオサキシトキシン (neoSTX)
OH	H	OSO_3^-	$CONH_2$	c ゴニオトキシン-1 (GTX_1)
H	H	OSO_3^-	$CONH_2$	d ゴニオトキシン-2 (GTX_2)
H	OSO_2^-	H	$CONH_2$	e ゴニオトキシン-3 (GTX_3)
OH	OSO_3^-	H	$CONH_2$	f ゴニオトキシン-4 (GTX_4)
H	H	H	$CONHSO_3^-$	g ゴニオトキシン-5 (GTX_5)
OH	H	H	$CONHSO_3^-$	h ゴニオトキシン-6 (GTX_6)
H	H	OSO_3^-	$CONHSO_3^-$	i エピゴニオトキシン-8 (epiGTX$_8$)
H	OSO_3^-	H	$CONHSO_3^-$	j ゴニオトキシン-8 (GTX_8)
OH	H	OSO_3^-	$CONHSO_3^-$	C_3
OH	OSO_3^-	H	$CONHSO_3^-$	C_4
H	H	H	H	k デカルバモイルサキシトキシン (dcSTX)
H	H	OSO_3^-	H	l デカルバモイルゴニオトキシン-2 (dcGTX$_2$)
H	OSO_3^-	H	H	m デカルバモイルゴニオトキシン-3 (dcGTX$_3$)

図 5■麻痺性貝毒群の化学構造

Fig. 5 Chemical structure of paralytic shellfish poison group

a saxitoxin (STX)
b neosaxitoxin (neo STX)
c goniotoxin -1 (GTX_1)
d goniotoxin -2 (GTX_2)
e goniotoxin -3 (GTX_3)
f goniotoxin -4 (GTX_4)
g goniotoxin -5 (GTX_5)
h goniotoxin -6 (GTX_6)
i epigoniotoxin -8 (epi GTX_8)
j goniotoxin -8 (GTX_8)
k decarbamoil saxitoxin (dc STX)
l decarbamoil goniotoxin -2 (dc GTX_2)
m decarbamoil goniotoxin -3 (dc GTX_3)

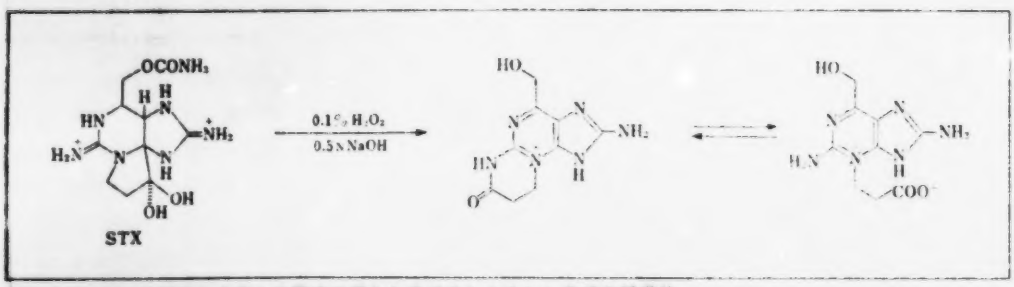


図 6■アルカリ性過酸化水素との反応で得られるサキシトキシンの蛍光誘導体

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Fig. 6 Fluorescent derivatives of saxitoxin obtained by the reaction with alkaline hydrogen peroxide

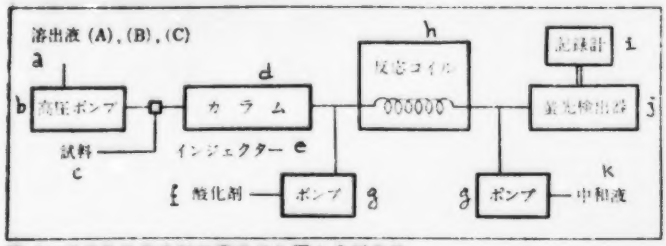

図 7 ■麻痺性貝毒分析装置の流れ図と分析条件

l 溶出液（流速，0.8 ml/分）：（A）GTX_8(C_2)，epi GTX_8(C_1)，C_3，C_4 分析用．1mM テトラブチルアンモニアーリン酸塩(酢酸で pH 5.7 に調整)．（B）GTX_1-GTX_6，dcGTX$_2$，dcGTX$_3$ 分析用．2mM 1-ヘプタンスルホン酸ナトリウム塩，10mM リン酸アンモニア緩衝液（pH 7.2）．（C）STX，neoSTX，dcSTX 分析用．溶液 B：アセトニトリル 90：10．

m カラム：Develosil C-8-5（4.6×250mm），酸化剤（流速，0.4 ml/分）：7mM 過ヨウ素酸，50mM リン酸ナトリウム緩衝液（pH 9.0），反応コイル：テフロンチューブ（内径，0.5 mm，長さ 10m），65℃，中和液（流速，0.4 ml/分）：0.5 M 酢酸，検出：ex. 330 nm，em. 390 nm．

Fig. 7 Flow chart of paralytic shellfish poison analyzer and analysis conditions

a eluate
b high pressure pump
c sample
d column
e injector
f acidifying agent
g pump
h reaction coil
i recorder
j fluorescence detector
k neutralized solution

l eluate (flow rate, 0.8 mL/min): (A) for GTX_8 (C_2), epi GTX_8 (C_1), C_3, C_4 analysis. 1 mM tetrabutyl ammonia-phosphate (adjusted to pH 5.7 with acetic acid). (B) for GTX_1-GTX_6, dc GTX_2, dc GTX_3 analysis. 2 mM 1-heptane sulfonic acid sodium salt, 10 mM ammonium phosphate buffer solution (pH 7.2). (C) for STX, neo STX, dc STX analysis. Solution B: acetonitrile 90:10

m column: Develosil C-8-5 (4.6 X 250 mm), oxidizing agent (flow rate 0.4 mL/min): 7 mM periodic acid, 50 mM sodium phosphate buffer solution (pH 9.0), reaction coil: teflon tube (inner diameter 0.5 mm, length 10 m), 65°C, neutralization solution (flow rate 0.4 mL/min): 0.5 M acetic acid, detection: ex. 330 nm, em. 390 nm.

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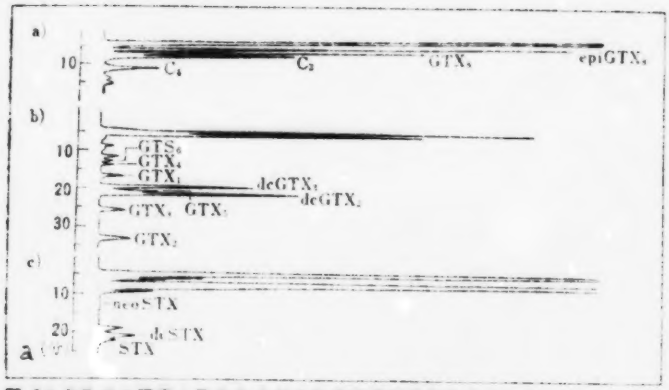

図 8■タスマニア産ムラサキイガイ中の麻痺性貝毒群の分析例

Fig. 8 Example of analysis of paralytic shellfish poison group in mussels from Tasmania

a min

p. 405

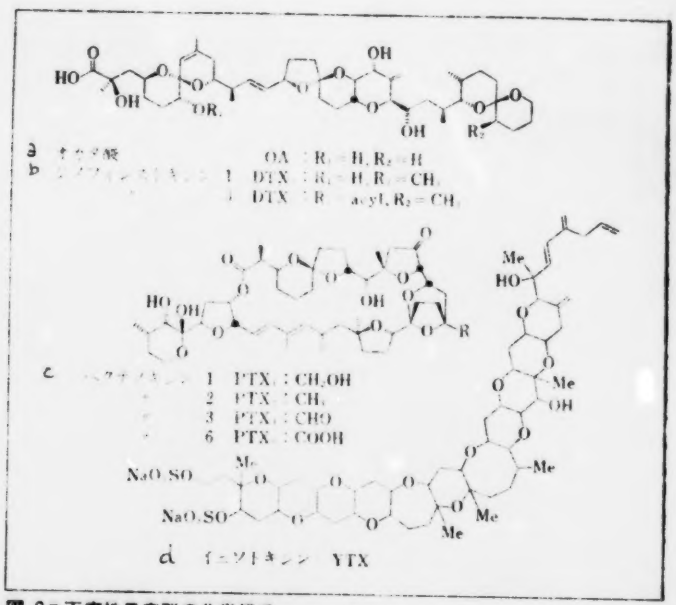

図 9■下痢性貝毒群の化学構造

Fig. 9 Chemical structure of the paralytic shellfish poison group

a okadaic acid
b dinophysis toxin
c pectenotoxin
d yessotoxin

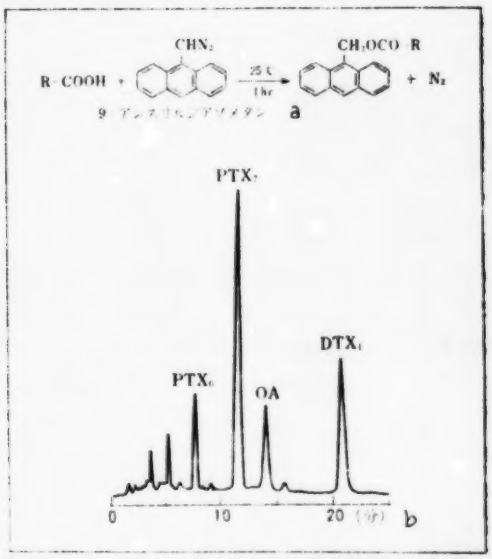

図 10■カルボキシル基を有する下痢性貝毒群と 1-アンスリルジアゾメタンとの反応および生成した螢光化合物のクロマトグラム

c クロム：Develosil ODS-5（野村化学，4.6×250 mm），溶媒：アセトニトリル-メタノール-水（8：1：1），流速：1.1 ml/分，検出：ex. 365 nm，em. 412 nm. OA：オカダ酸，DTX_1：ジノフィシストキシン-1，PTX_6：ペクテノトキシン-6，PTX_7：ペクテノトキシン-7（構造未定のため図9に示してない）．

Fig. 10 Reaction between the paralytic shellfish poison group having carboxyl radicals and 1- anthryl diazomethane, with the chromatogrammes of the fluorescent compounds produced

a 9- anthryldiazomethane
b min

c column: Develosil ODS-5 (Nomura Kayaku, 4.6 X 250 mm), solvent: acetonitril - methanol - water (8:1:1), flow rate: 1.1 mL/min, detection: ex. 365 nm, em. 412 nm. OA: okadaic acid, DTX_1: dinophysistoxin -1, PTX_6: pectenotoxin-6, PTX_7: pectenotoxin-7 (not shown in Fig. 9 as structure is undetermined).

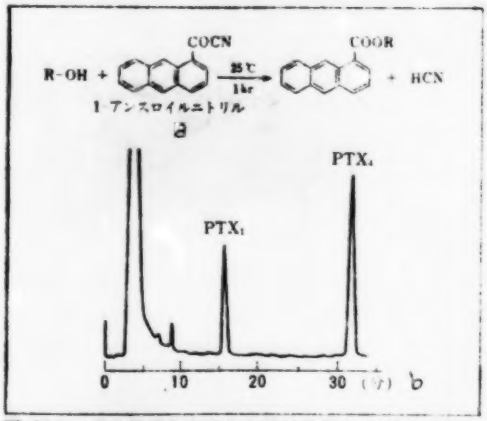

図 11■一級水酸基を有する下痢性貝毒と 1-アンスロイルニトリルとの反応および生成した化合物のクロマトグラム

c カラム：Develosil ODS (野村化学, 4.6×250 cm), 溶媒：アセトニトリル-水 (8:2) 1.1 ml/分, 検出：ex. 365 nm, em. 465nm. PTX_1：ペクテノトキシン-1, PTX_4：ペクテノトキシン-4 (構造未定のため図 9 には示していない).

Fig. 11 Reaction between diarrhetic shellfish poison having first class hydroxyl groups and 1-anthroylnitrile together with the chromatogrammes of the compounds produced

a anthroylnitrile
b min

c column: Develosil ODS (Nomura Kagaku, 4.6 X 250 cm), solvent: acetonitrile - water (8:2) 1.1 mL/min, detection: ex. 365 nm, em. 465 nm. PTX_1: pectenotoxin -1, PTX_4: pectenotoxin - 4 (not shown in Fig. 9 as structure is undetermined)

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